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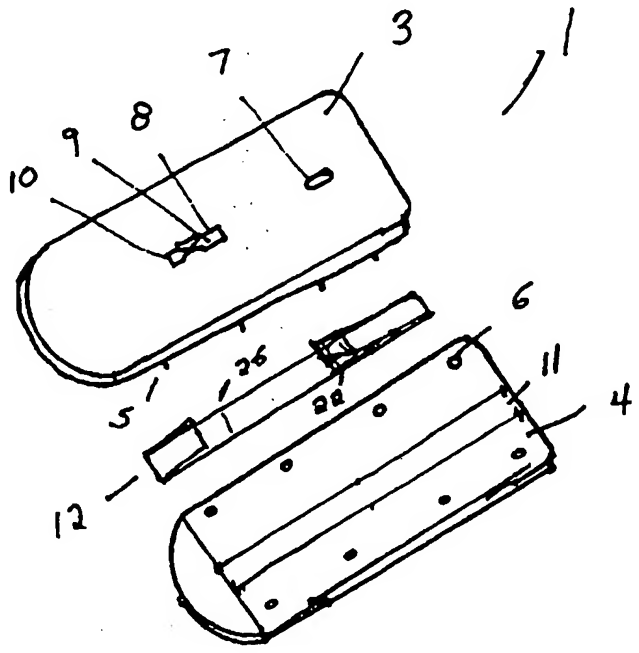
(54) Title: OPTIMIZING SENSITIVITY IN COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW TESTS

(57) Abstract

An immunoassay test device is disclosed that allows for easy identification of positive reactions. The test device provides a simplified, sensitive and specific test procedure for the determination and detection of an immunologically reactive analyte in an aqueous sample. The test device comprises a plastic enclosure (2) having a top section (3) and a bottom section (4). The top section (3) of enclosure (2) has an opening (7) for receiving sample and an opening (8) for visualizing test results (9) and for visualizing a control result (10). Bottom section (4) comprises a tray (11) into which fits a capture membrane (12). The capture membrane (12) aligns a capture antibody on a solid surface so that the capture capability of the capture antibody is not sterically hindered.



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OPTIMIZING SENSITIVITY IN COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW TESTS

DESCRIPTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention discloses a colorimetric immunoassay test system.

2. Description of the Prior Art

The past twenty years have seen significant advances in rapid immunodiagnostic techniques. These methods include radio-immunoassay, counter electrophoresis, ELISA and membrane strip tests utilizing reagents tagged with a colorimetric label (e.g. gold, colored latex, etc.). Rapid diagnostic tests utilizing gold labeled antibody to visualize the test results on antibody coated membranes has evolved to be the test-of-choice in cases where manual, fast, and inexpensive, non-instrument test formats are required. The major area of concern, when utilizing the rapid gold test format, is the relative lack of sensitivity when compared to tests such as ELISA, which utilize enzyme enhanced reactions, multiple incubations steps, and prolonged time.

Over the years, there have been many gold immunoassay test systems developed to show positive results in the face of particular antigens or antibodies. These assay systems utilize a colloidal gold particle concentration immunoassay to achieve sensitive and selective detection of biological materials.

Antibodies specific to the agent of interest are conjugated to colloidal gold particles. Colloidal gold consists of discrete, electron-dense, red-colored particles ranging from 10 ng to 100 ng in diameter with a very high extinction coefficient. When concentrated on solid surfaces, these particles can be visually observed. Labeled antibodies can be easily lyophilized and reconstituted without losing activity or specificity.

If antigen is present in a collected sample, an immune complex will form between the colloidal gold-labeled detector antibody (Ab) and the antigen (Ag). The test sample is used to reconstitute a dried colloidal gold labeled antibody and the resulting mixture (antibody and test antigen) ascends chromatographically up a strip that ha been layered with a capture antibody or directly through an antibody coated membrane. The presence of a red stripe or a dot is indicative of a positive test. The test strips contain a positive control to ascertain that the test is working properly.

U.S. Patent No. 5,514,602 (Brooks, Jr. et al.) discloses a method of producing a metal sol reagent containing colloidal metal particles. A metal containing solution is reduced under optimized pH conditions to produce metal sol particles of a preselected size. The particles are coated with a coupling compound, and then bound with at least one selected immunochemically reactive component. Particles having different immunochemical specificities are also mixed to produce reagents having multiple selected immunochemical specificities.

U.S. Patent No. 5,384,265 (Kidwell et al.) discloses

contacting a sample which may contain an analyte with a biomolecule which is bound to a catalytically active colloidal metal particle, to obtain an analyte-biomolecule-colloidal metal particle complex, separating the analyte-biomolecule-colloidal metal particle complex from the sample, reacting the analyte-biomolecule-colloidal metal particle complex with hydrazine in the presence of lucigenin at a pH of 8 to 11; and detecting light generated by the reaction of the analyte-biomolecule-colloidal metal particle complex in the presence of lucigenin.

U.S. Patent No. 5,294,369 (Shigekawa et. al.) discloses a gold sol coated with alkanethiols and alkanethiol derivatives which provide groups on the sol available for the linking of binding moieties such as antibodies, antigens or ligands to the gold sol.

U.S. Patent No. 5,334,538 (Parker et al.) discloses a gold sol immunoassay system and device. The gold sol bead is held in a funnel member. Antibodies are associated with the gold sol bead. When the sample contacts the gold sol, it dissolves the bead. A second antibody is impregnated on an immunosorbent surface. When the dissolved gold sol passes this surface, any antigen already reacted with the first antibody present reacts with the second antibody forming a gold: first antibody: antigen: second antibody: immunosorbent complex. The gold sol acts as the visible label.

U.S. Patent No. 5,120,643 (Ching et al.) discloses a process for immunochromatography with colloidal particles. The method comprises the steps of: contacting a chromatographic medium with the test sample, with the medium comprising at least two reaction

sites. The first reaction site comprises a dried solution or a labeled specific binding reagent in the presence of a meta-soluble protein, and a second reaction site comprising an immobilized specific binding reagent in relation to the presence or amount of the analyte in the test sample. The labeled reagent is solubilized and at least a portion of the labeled reagent is transported to the second reaction site, with the binding dependent upon the presence or amount of the analyte in the test sample. The labeled reagent is detected at the second site to determine the presence or amount of the analyte in the test sample.

U.S. Patent No. 5,079,172 (Hari et al.) discloses a method and kit for detecting the presence of antibodies using gold-labeled antibodies. Microspheres coated with an antigen reactive with the first antibody are reacted with the first antibody from serum or other sources. The gold-labeled antibody is reacted with the first antibody antigen complex on the microsphere and detected. Preferably, the gold particles are detected using an electron microscope.

PCT/US95/04547 describes the use of soluble submicron particles (dendrimers) that are labeled with antibodies to coat membranes. The assay procedures described are flow through EIA and fluorescence immunoassays requiring washing steps in order to obtain a response.

Currently, most lateral flow and flow-through gold immunoassays utilize antibody bound directly to porous membranes or to particles such as glass or latex to capture antigen-antibody-

The original tests developed by New Horizons Diagnostics utilized primarily 20 nm gold particles to tag the antibodies. This resulted in a sensitivity level of about 50 ng for botulism toxin and around 10^5 organisms/cc for the quantity of specific bacteria that could be detected. 20 nm particles were used initially because of the stability of the gold tagged antibody conjugate and the low background signal (if any) it gave in negative test samples. Ideally, it is better to label with a much larger gold colloid (anywhere between 50 nm to 100 nm) to increase the sensitivity of test results. However, the problem with using larger colloidal gold complexes for tagging has been the increase in non-specificity, which creates false positive reactions.

SUMMARY OF THE INVENTION

The present invention proposes a much more sensitive immunoassay test, which is easier to use and interpret. The entire test is conducted on a test strip, and the detection antibody is preferably a FAB fragment that has been labeled with a 50 - 100 nm gold particle and immobilized on a test pad. The invention provides a simplified, sensitive and specific test procedure for the determination and detection of an immunologically reactive analyte in an aqueous sample.

Specifically, the present invention discloses a capture membrane to align and secure capture antibodies on a solid surface so that the immunological activity of the capture antibody is not sterically hindered. Consequently, optimal binding capacity is

achieved, resulting in a minimum loss in binding activity between the capture antibody and the ligand. In one preferred embodiment, dendrimers are used to secure the capture antibodies to a test strip upon which a sample is placed.

In another embodiment of the invention, the site of the capture membrane is protein A or protein G. Alternatively, the capture membrane site may be lectin receptors, to which no capture antibodies are applied. Antispecies antibodies may be used, particularly in combination with the dendrimers or protein G.

The present invention also discloses an improved gold immunoassay test system which uses larger gold colloids for tagging antibodies (anywhere between 50 nm to 100 nm) to increase the sensitivity of test results, (i.e. each specific antibody that reacts, delivers more gold complex to the antibody capture membrane) without any residual non-specific activity. The elimination of non-specific background activity problems results from the use of blocking chemistries which inhibit non-specific reactions without altering the desired specific reaction and the use of specifically designed recombinant FAB antibodies.

FAB antibodies are unlike whole antibodies in that their FC or constant regions are eliminated. It is this region of the antibody molecule that often causes problems with non-specificity.

In this invention, the FAB antibodies for detecting a positive result are attached to gold colloidal masses, in the range of 50-100 nm. These gold-FAB antibody complexes are positioned on a test strip, downstream from where the antigenic sample is applied.

The gold FAB-antibodies may be attached to microspheres, thereby allowing for more antibodies to be located at the reagent site.

Additionally, other metals or dyes may be attached to the FAB antibodies, in place of the gold particles.

Further downstream from both the antigenic or ligand sample and the gold-FAB sample is a set of antibodies, specific for the gold-FAB antibody-antigen or antibody-ligand complex. These antibodies serve to concentrate the complex in one location, thereby allowing for a red stripe to appear on a set section of the test strip when there is a positive reaction.

The capture antibodies are located further downstream from the reagent and the sample site.

Dendrimers are one way of attaching the capture antibodies to the test strip. Dendrimers are three dimensional, tree-like polymers. The dendrimers have a small size, good solubility, higher segmental densities, interior void space, and lower viscosities. Dendritic polymers can be constructed by both divergent and convergent synthetic methods. The divergent synthesis starts from a center core, and then grows each layer in a stepwise fashion, while the convergent method assembles exterior end groups and dendrons first before being coupled onto a core. Each re-iteration or layer is defined as a generation. The more layers there are in the dendrimeric structure, the more rigid the dendrimers molecule itself becomes. Up to about G8, a dense packing stage also occurs, where beyond this point, monomers can no longer react with every

surface group quantitatively due to the stearic effect. In the case of immunoassays, the rigid, spherical protein-like sizes controls the antibody binding direction, while the exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion on a membrane surface. This property allows the dendrimers-antibody conjugate to self-assemble to its best binding orientation. As a result, the sensitivity and reproducibility of the immunoassay in the detection of different bio-threat agents has been significantly improved, while the assay time has been shortened. In addition due to the low viscosity nature and self assembling capabilities of dendrimers-antibody conjugates, the process for producing these tickets has been simplified, and has reduced lot to lot variability.

Dendrimers have the advantage, that they can be synthesized with an exactly uniform molecular weight, whereas the conventional polymers always have a particular molecular weight distribution. In dendrimers with particular functinal groups can be manufactured with a defined number of such reactive groups.

The coupling of antibodies or other ligands to outer surfaces of dendrimers (for example polyamidoamine [PAMAMS] dendrimers) can be done by various well known chemistries which describe Carbon-sulfur, carbon-oxygen, and carbon-nitrogen coupling procedures. The exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion onto the surface of a membrane. The structural composition of the dendrimer controls the spatial arrangement of the attached antibody

molecules. This assures the optimal binding activity of the immobilized capture antibody.

Brief Description of the Drawings

The above and other objects, features and advantages of the present invention will become more readily apparent from the following description, reference being made to the accompanying drawings in which:

FIG. 1 is an overall view of the immunoassay test system;

FIG. 2 is an exploded view of the immunoassay test system;

FIG. 3 is a side view of the immunoassay test strip;

FIG. 4 is an overhead view of the immunoassay test strip; and

Fig. 5 is a schematic drawing of the antigenic-antibody reaction.

Description of the Preferred Embodiment

Referring to Figures 1-5, the immunoassay test system 1 comprises an enclosure 2, which is preferably plastic. This plastic enclosure comprises a top section 3 and a bottom section 4 which are held together by male 5 and female 6 peg joints.

The top section 3 of the enclosure 2 has an opening 7 for placing a sample. There is also an opening 8 to visualize the test results 9 and the control results 10.

The bottom section 4 comprises a tray 11 into which fits a test strip 12. The test strip 12 preferably has a membrane support 13. The membrane support 13 may be comprised of plastic, cardboard, or any other rigid material. On top of the membrane support 13 is a testing layer 14, preferably made out of nitrocellulose. On top of the nitrocellulose or testing layer 14 are the areas to which the appropriate reagents or samples are applied or affixed. The nitrocellulose/testing layer is affixed to the membrane support 13 by an adhesive 31.

At one end of the test strip 12 is the sample site 15 to which the sample is to be applied. This sample site 15 may have another nitrocellulose or adsorbent sample pad 23 residing on top of the testing layer, to which the sample is transferred. The sample may be in the form of an antigen or ligand 16 in a fluid.

The list of target ligands and anti-ligands which potentially may be detected or determined includes antigens and ligands found in animal body fluids, as well as antigens associated with bacteria, parasites, fungi, viruses, toxins, anthrax, etc. Moreover, therapeutic drugs and controlled substances having small molecules, such as, for example, theophylline, may be detected or determined using the present invention.

The sample travels downstream from the sample site 15 to the gold immunoassay site 18 where FAB antibody coated gold

sol particles 19 reside. The gold particles 19 attached to the FAB antibodies 20 are preferably larger than 20 nm, more preferably in the range of about 50 to 100 nm, and most preferably in the range of from about 70 to 90 nm. Larger particles may also be used wherein a number of FAB antibodies 20 are attached to the gold particle 19. The gold sol labeled FAB antibodies 21 are dried and deposited on the strip 12.

The metal sol particles to be used in accordance with the present invention may be prepared by coupling an immunologically reactive substance directly to the gold particle. Additionally, the labeled component may be prepared by coupling the substance to the particle using a biotin/avidin linkage. In this latter regard, the substance may be biotinylated and the metal containing particle coated with an avidin compound. The biotin on the substance may then be reacted with the avidin compound on the particle to couple the substance and the particle together. In another alternative form of the invention, the labeled component may be prepared by coupling the substance antibody to a carrier such as bovine serum albumin (BSA), and using this to bind to the metal particles.

The metal sol particles to be used in accordance with the present invention may be prepared by methodology which is well known. For instance, the preparation of gold sol particles is disclosed in an article by G. Frens, *Nature*, 241, 20-22 (1973). Additionally, the metal sol particles may be metal or metal compounds or polymer nuclei coated with metals or metal compounds,

as described in U.S. Pat No. 4,313,734. Other methods well known in the art may be used to attach the gold particles to the FAB antibodies. The metal sol particles may be made of platinum, gold, silver, or copper or any number of metal compounds which exhibit characteristic colors.

Similarly, the antibodies do not necessarily have to be attached to a metal sol particle, but may instead be attached to a dye with an extinction coefficient equal to or greater than gold. The metal sol particles or dyes should have a high extinction coefficient equal to or greater than gold.

There are a number of ways in which the gold labeled FAB antibodies **21** may be deposited on the strip **12**.

In an alternative and preferred embodiment, the gold labeled FAB antibodies are deposited and dried on a rectangular or square or adsorbent FAB antibody pad **22**, the pad preferably about .25" x .25"or less. This FAB antibody pad **22** is positioned downstream from where the sample is applied on the strip **12**. Preferably, the FAB antibody pad **22** fits underneath the distal end **24** of the sample pad **23**.

In yet another embodiment of the invention, the antibodies are attached to microspheres. This has the effect of increasing the number antibodies in a given area.

The process for attaching the antibodies to the microsphere(s) begins with the use of protein reactive microspheres (MX-Covaspheres® of diameter .5 micrometers or .9 micrometers purchased

from Duke Scientific Corporation, Pal Alto, California 94303. The microspheres may also be purchased from other suppliers, as well). The binding is at the amino groups of the protein. They were then coated with various antibodies. A suspension of the spheres are mixed after sonication with the antibodies in water or in a phosphate buffer solution, after which they are incubated at room temperature for 10-75 minutes. The mixture is then centrifuged in an Eppendorf microcentrifuge and the pellets containing the antibody-linked microspheres are suspended in a buffer containing 1-5% by wt/volume bovine serum albumin (BSA) for 1 hour at room temperature. The BSA blocked any unreacted surfaces of the microspheres. After one more centrifugation, at 10,000 for 10 minutes, the spheres were resuspended in the above buffer (TBS with 5% BSA) and stored at 4 degrees C. before using.

The solid phase particles may comprise any one of known, water dispersable particles, such as, for example, the polystyrene latex particles discloses in U.S. Patent No. 3,088,875. Such solid phase materials simply consist of suspensions of small, water-insoluble particles to which antibodies are able to bind. Suitable solid phase particles are also disclosed, for example, in U.S. Patent Nos. 4,184,849; 4,486,530; and 4,636,479.

The solid phase particles useful in connection with the invention may comprise, for example, particles of latex or of other support materials such as silica, agarose, glass, polyacrylamides, polymethyl methacrylates, carboxylate modified latex and Sepharose. Preferably, the particles will vary in size from about 0.2 microns

to about 10 microns. In particular, useful commercially available materials include .99 micron carboxylate modified latex, cyanogen bromide activated Sepharose beads (Sigma), fused silica particles (Ciba Corning, lot #6), isothiocyanate glass (Sigma), Reactogel 25DF (Pierce) and Polybead - carboxylate monodisperse microspheres. In accordance with the invention, such particles may be coated with a layer of FAB antibodies coupled thereto in a manner known per se in the art to present the solid phase component.

If the sample contains an antigen or ligand **16** to which the gold FAB antibodies **21** react, there is a antigenic-antibody bonding between the sample and the gold FAB antibodies **21**. The antigen-gold FAB antibody complex **25** continues to migrate along the capture site **26** where another antibody is attached to a region of the strip **12**.

The antibodies **27** supported by the dendrimers **32** are designed to react specifically to the antigen, effectively forming an antibody-antigen-gold FAB antibody sandwich **29** if there is a positive reaction. If there is a negative reaction, no "sandwich" is formed, and the unreacted ligand proceeds to the end of the strip **12** wherein an absorbent pad **30** absorbs the fluid and unreacted labelled-antibody proceeds to the end of the strip **12**.

To prepare the capture site **26**, antibodies are bound to dendrimers prior to their placement on the strip **12**. This is then layered on the strip and dried.

In performing the testing of an antigen or ligand, it should

be noted that if there is a positive test result, the antigens or ligands will attach to the gold FAB antibodies as it migrates from the sample site, whereupon the antigens or ligands will attach to the antibodies attached to the dendrimers. At this point, the concentrated appearance of the gold particles appears as a red to purple line. If however, the ligands do not attach to the gold labeled antibodies, the antibodies will not be bound to the capture site 26.

2/12/98 JF. The use of dendrimers is disclosed in the copending application = Colloidal Colocin Flow Through and Lateral Flow Assay Utilizing Soluble Substrate Particles Filed June 12, 1998, Harvey Docket # NH-10

There are a number of other means in which antibodies may be attached or positioned at the capture site. Instead of using dendrimers at the capture site, Protein A and/or Protein G from *Staphylococcus* cell wall may be deposited by conventional means to the test strip. Because of the unique chemistry of Protein A and/or Protein G, the capture antibodies laid down at the capture site are aligned such that the active or binding ends are facing outward from the protein surface. Protein A and Protein G have a strong anti-FC activity. Consequently, FAB antibodies tagged with gold must be used at the reagent site; otherwise, false positive reactions may result as "loose" gold antibodies migrate along the test strip.

In another embodiment of the invention, a species-antispecies antibody combination is laid down on the test strip. For example, a goat antirabbit antibody is laid down on the test strip. The rabbit antibody is attached or bound to the goat anti-rabbit antibody. The rabbit antibody may be non-specific, so that any antigen that migrates along the test strip will be captured by the rabbit antibody. If the colorimetric tagged antibody is attached to

the antigen, a positive result will be appear in the form of an indicator line. It should be noted that the anti-species antibody may be attached to the test strip by a dendrimeric arrangement.

In yet another alternative embodiment of the invention at the capture site is to have lectin receptors. This lectin layer will bind the antigen as it migrates along the length of the test strip. In the preferred embodiment, the lectin may be bound to a dendrimer to optimize its binding capability.

The superior sensitivity of this test format allows for detection of amounts of antigen or ligand, measured in picograms.

Additionally, this test can also have positive and negative control lines. The positive control line has an anti-Fab substance or antibody laid down at the appropriate spot on the strip, downstream from the sample test site and from the FAB reagent site. This line should always appear when FAB antibodies are used in the test. If the positive control is negative, then the test is invalid.

The negative control can use any nonrelated antibody to coat the strip. There should be no capture of the antigen or ligand by the non-related antibody. If the negative control is positive, i.e., a line appears, than the test is invalid.

Many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood within the scope of the appended claims the invention may be protected otherwise than as specifically described.

What is claimed is:

1) A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a testing layer, said testing layer comprised of an absorbent material;

a sample site at one end of the testing layer, to which a ligand is applied;

a reagent-colorimetric immunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of antibodies attached to colorimetric particles thereby forming colorimetric labeled antibodies, said colorimetric labeled antibodies residing at said reagent-colorimetric immunoassay site;

a capture site, said capture site comprising means to retain ligand-colorimetric labeled antibody complexes in optimal alignment, said means comprising capture antibodies and binding means to align said capture antibodies so that active ends of said capture antibodies face away from an outwardly facing surface of said testing layer;

wherein said ligand, when placed on said sample site, migrates to the reagent-colorimetric-immunoassay site, and then migrates to the capture site, such that if said ligand reacts positively with the colorimetric labeled antibodies, said ligand-colorimetric labeled antibody complexes migrate to the capture site, and a positive response will appear as a colorimetric line at the capture site when an antibody-ligand-colorimetric labeled antibody

sandwich is formed.

2) The immunoassay test system of claim 1, wherein said antibodies at said reagent-colorimetric immunoassay site are FAB antibodies.

3) The immunoassay test system of claim 1, wherein said testing layer is comprised of nitrocellulose.

4) The immunoassay test system of claim 1, further comprising a support backing layer upon which the testing layer resides.

5) The immunoassay test system of 4, wherein said support backing layer is made of a rigid material, said material being selected from the group consisting of cardboard or plastic.

6) The immunoassay test system of claim 1, wherein said testing layer is fixed to said backing layer by an adhesive.

7) The immunoassay test system of claim 1, wherein said colorimetric particles are selected from the group consisting of gold particles, silver particles, platinum particles, copper particles and encapsulated dyes.

8) The immunoassay test system of claim 7, wherein the colorimetric particles are gold sol particles.

9) The immunoassay test system of claim 8, wherein said gold sol particles may range in size from about 20 ng to about 100 ng.

10) The immunoassay test system of claim 9, wherein said gold sol particles may range in size from about 50 ng to about 90 ng.

11) The immunoassay test of claim 1, further comprising microspheres, wherein said colorimetric labeled antibodies are attached to said microspheres.

12) The immunoassay test system of claim 1, wherein said binding means which aligns said capture antibodies is selected from the group consisting of Protein A, Protein G, and any other substance which binds said antibodies at said capture site.

13) The immunoassay test system of claim 1, wherein said antibodies at the reagent-colorimetric immunoassay site are FAB antibodies.

14) The immunoassay test system of claim 13, wherein said binding means which aligns said capture antibodies is selected from the group consisting of Protein A and Protein G.

15) The immunoassay test system of claim 1, wherein said ligand is an antigen.

16) The immunoassay test system of claim 1, wherein the ligands and anti-ligands which may be detected are selected from the group consisting of antigens and ligands found in animal body fluids, antigens associated with bacteria parasites, fungi, viruses therapeutic drugs and controlled substances having small molecules.

17) The immunoassay test system of claim 12, wherein said dendrimers for aligning said capture antibodies are starburst dendrimers.

18) The immunoassay test system of claim 1, further comprising an enclosure,

said enclosure comprising a top section, said top section having openings for placing a sample and for visualizing the test results; and

a bottom section said bottom section having a tray into which fits a test strip.

19) The immunoassay test system of claim 18, wherein said enclosure is comprised of plastic.

20) The immunoassay test system of claim 1, wherein said absorbent material allows for absorption of a liquid sample and has capillary action capability, thereby allowing the sample to migrate along the length of the test strip.

21) The immunoassay test system of claim 1, further comprising a sample pad positioned on top of the testing layer at the sample site upon which the sample is added.

22) The immunoassay test system of claim 1, further comprising a FAB antibody pad positioned on top of the testing layer at the reagent-gold immunoassay site at which the FAB antibodies may react with an appropriate ligand.

23) The immunoassay test system of claim 1, further comprising an absorption pad positioned on top of the testing layer downstream from the capture site to absorb any excess fluid.

24) The immunoassay test system of claim 1, further comprising a negative control line positioned on said test strip, such that if said negative control line shows a positive result in the performance of a test, said test is invalid.

25) The immunoassay test system of claim 1, further comprising a positive control line positioned on said test strip such that if said positive control line shows a negative result in the performance of a test, said test is invalid.

26) The immunoassay test system of claim 1, further comprising a positive control line and a negative control line positioned on said test strip such that if said positive control line shows a

negative result in the performance of a test, or said negative control line shows a positive result in the performance of said test, said test is invalid.

27) A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a support backing layer;

a testing layer, said testing layer comprised of an absorbent material;

a sample site at one end of the testing layer, to which a ligand is applied;

a reagent-colorimetric immunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of FAB antibodies attached to colorimetric particles thereby forming colorimetric labeled FAB antibodies, said colorimetric labeled FAB antibodies residing at said reagent-colorimetric immunoassay site;

a capture site, said capture site comprising means to retain ligand-colorimetric labeled antibody complexes, said means to retain said ligand-colorimetric labeled antibody complexes being a lectin layer attached to said test strip;

wherein said ligand, when placed on said sample site, migrates to the reagent-colorimetric-immunoassay site, and then migrates to the capture site, such that if said ligand reacts positively with the colorimetric labeled FAB antibodies, said ligand-colorimetric labeled FAB antibody complexes migrate to the

capture site, and a positive response will appear as a red line at the capture site when an antibody-ligand-colorimetric labeled FAB antibody sandwich is formed.

28) A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a support backing layer;

a testing layer, said testing layer comprised of an absorbent material;

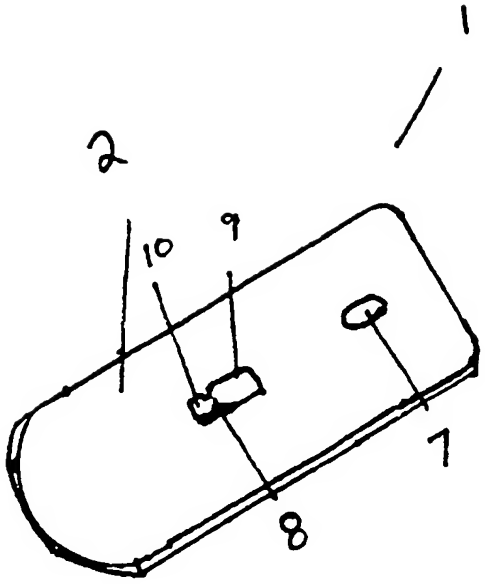
a sample site at one end of the testing layer, to which a ligand is applied;

a reagent-colorimetric immunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of antibodies attached to colorimetric particles thereby forming colorimetric labeled antibodies, said colorimetric labeled antibodies residing at said reagent-colorimetric immunoassay site;

a capture site, said capture site comprising means to retain ligand-colorimetric labeled antibody complexes in optimal alignment, said means to retain said ligand-colorimetric labeled antibody complexes being a species antibody-antispecies antibody layer attached to said test strip, said capture site further comprising a means of attaching the antispecies antibodies to said test strip, wherein said species antibodies are reacted and attached to said antispecies antibodies; and

wherein said ligand, when placed on said sample site,

migrates to the reagent-colorimetric-immunoassay site, and then migrates to the capture site, such that if said ligand reacts positively with the colorimetric labeled antibodies, said ligand-colorimetric labeled FAB antibody complexes migrate to the capture site, and a positive response will appear as a red line at the capture site when an antispecies antibody species antibody-ligand-colorimetric labeled antibody sandwich is formed.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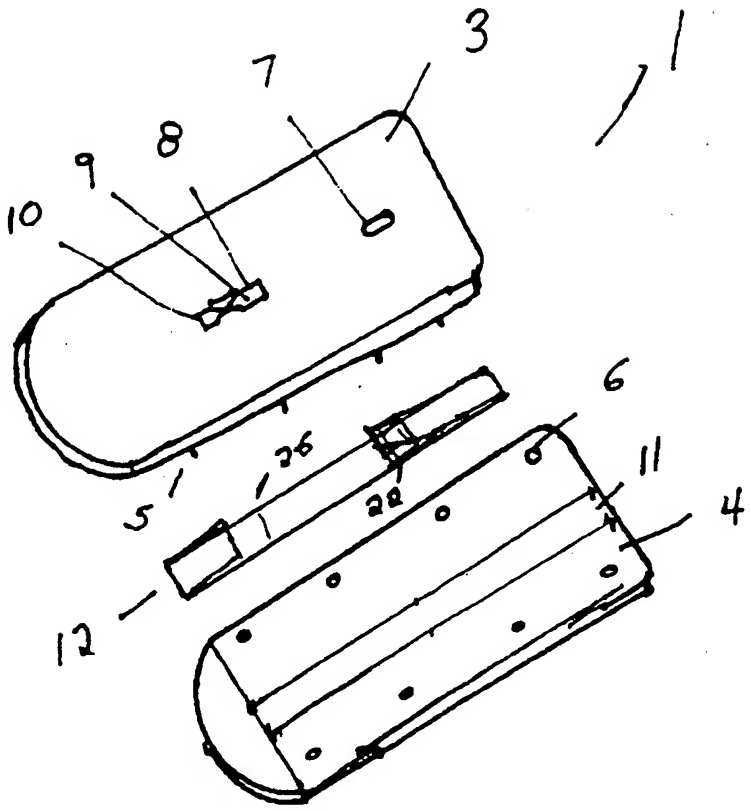
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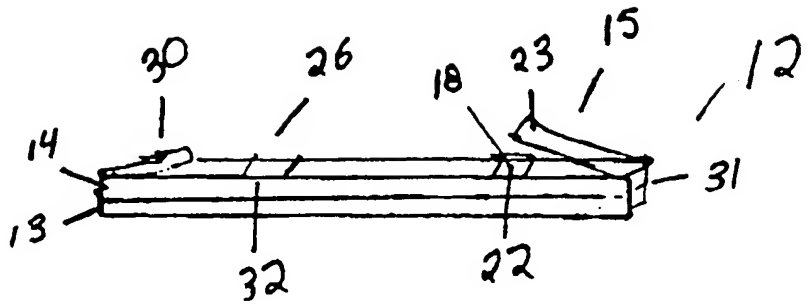
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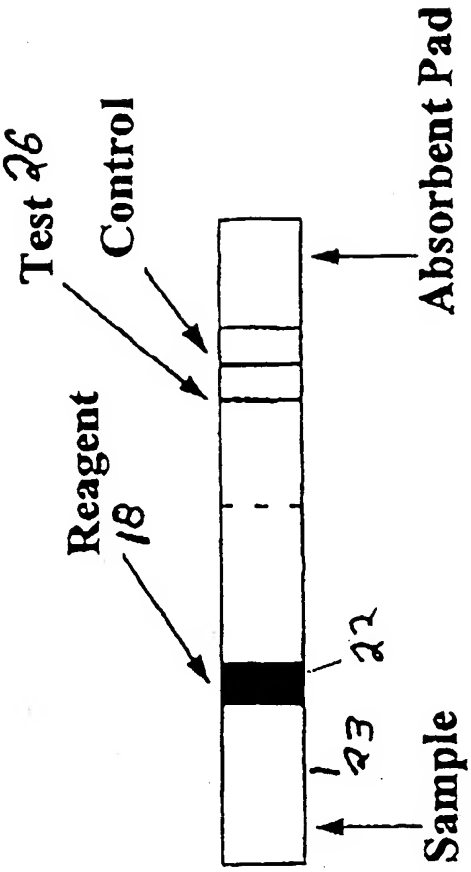
Lateral Flow Device



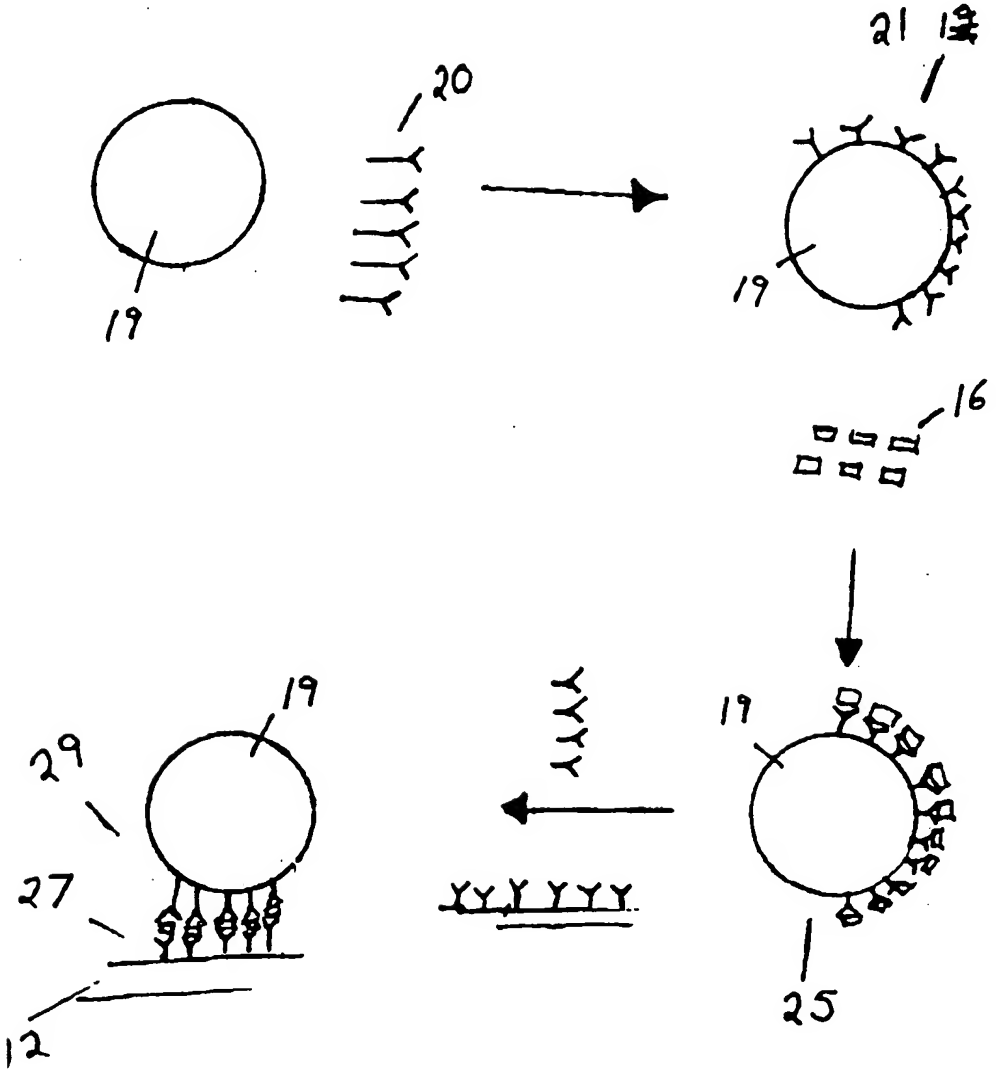


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13284

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/558
US CL :436/514

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,602,040 A (MAY et al) 11 February 1997, see entire document.	1-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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INTERNATIONAL SEARCH REPORT

International application No.
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B. FIELDS SEARCHED
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CORRECTED VERSION*

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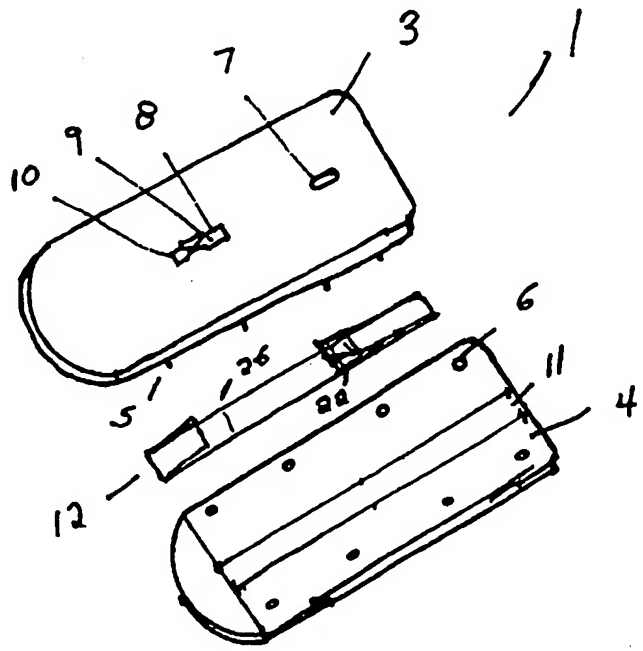
(54) Title: OPTIMIZING SENSITIVITY IN COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW TESTS

(57) Abstract

An immunoassay test device is disclosed that allows for easy identification of positive reactions. The test device provides a simplified, sensitive and specific test procedure for the determination and detection of an immunologically reactive analyte in an aqueous sample. The test device comprises a plastic enclosure (2) having a top section (3) and a bottom section (4). The top section (3) of enclosure (2) has an opening (7) for receiving sample and an opening (8) for visualizing test results (9) and for visualizing a control result (10). Bottom section (4) comprises a tray (11) into which fits a capture membrane (12). The capture membrane (12) aligns a capture antibody on a solid surface so that the capture capability of the capture antibody is not sterically hindered.



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OPTIMIZING SENSITIVITY IN COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW TESTS

DESCRIPTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention discloses a colorimetric immunoassay test system.

2. Description of the Related Art

The past twenty years have seen significant advances in rapid immunodiagnostic techniques. These methods include radioimmunoassay, counter electrophoresis, ELISA and membrane strip tests utilizing reagents tagged with a colorimetric label (e.g. gold, colored latex, etc.). Rapid diagnostic tests utilizing gold labeled antibody to visualize the test results on antibody coated membranes has evolved to be the test-of-choice in cases where manual, fast, and inexpensive, non-instrument test formats are required. The major area of concern, when utilizing the rapid gold test format, is the relative lack of sensitivity when compared to tests such as ELISA, which utilize enzyme enhanced reactions, multiple incubations steps, and prolonged time.

Over the years, there have been many gold immunoassay test systems developed to show positive results in the face of particular antigens or antibodies. These assay systems utilize a colloidal gold particle concentration immunoassay to achieve sensitive and selective detection of biological materials. Antibodies specific to the agent of interest are conjugated to colloidal gold particles. Colloidal gold consists of discrete, electron-dense, red-colored particles ranging from 10 ng to 100 ng in diameter with a very high extinction coefficient. When concentrated on solid surfaces, these particles can be visually observed. Labeled antibodies can be easily lyophilized and reconstituted without losing activity or specificity.

If antigen is present in a collected sample, an immune complex will form between the colloidal gold-labeled detector antibody (Ab) and the antigen (Ag). The test sample is used to reconstitute a dried colloidal gold labeled antibody and the resulting mixture (antibody and test antigen) ascends chromatographically up a strip that ha been layered with a capture antibody or directly through an antibody coated membrane. The presence of a red stripe or a dot is indicative of a positive test. The test strips contain a positive control to ascertain that the test is working properly.

U.S. Patent No. 5,514,602 (Brooks, Jr. et al.) discloses a method of producing a metal sol reagent containing colloidal metal particles. A metal containing solution is reduced under optimized pH conditions to produce metal sol particles of a preselected size. The particles are coated with a coupling compound, and then bound with at least one selected immnunochemically reactive component. Particles having different immunochemical specificities are also mixed to produce reagents having multiple selected immunochemical specificities.

U.S. Patent No. 5,384,265 (Kidwell et al.) discloses contacting a sample which may contain an analyte with a biomolecule which is bound to a catalytically active colloidal metal particle, to obtain an analyte-biomolecule-colloidal metal particle complex, separating the analyte-biomolecule-colloidal metal particle complex from the sample, reacting the analyte-biomolecule-colloidal metal particle complex with hydrazine in the presence of lucigenin at a pH of 8 to 11; and detecting light generated by the reaction of the analyte-biomolecule-colloidal metal particle complex in the presence of lucigenin.

U.S. Patent No. 5,294,369 (Shigekawa et. al.) discloses a gold sol coated with alkanethiols and alkanethiol derivatives which provide groups on the sol available for the linking of binding moieties such as antibodies, antigens or ligands to the gold sol.

U.S. Patent No. 5,334,538 (Parker et al.) discloses a gold sol immunoassay system and device. The gold sol bead is held in a funnel member. Antibodies are associated with the gold sol bead. When the sample contacts the gold sol, it dissolves the bead. A second antibody is impregnated on an immunosorbent

surface. When the dissolved gold sol passes this surface, any antigen already reacted with the first antibody present reacts with the second antibody forming a gold: first antibody: antigen: second antibody: immunosorbent complex. The gold sol acts as the visible label.

U.S. Patent No. 5,120,643 (Ching et al.) discloses a process for imnunochromatography with colloidal particles. The method comprises the steps of: contacting a chromatographic medium with the test sample, with the medium comprising at least two reaction sites. The first reaction site comprises a dried solution of a labeled specific binding reagent in the presence of a meta-soluble protein, and a second reaction site comprising an immobilized specific binding reagent in relation to the presence or amount of the analyte in the test sample. The labeled reagent is solubilized and at least a portion of the labeled reagent is transported to the second reaction site, with the binding dependent upon the presence or amount of the analyte in the test sample. The labeled reagent is detected at the second site to determine the presence or amount of the analyte in the test sample.

U.S. Patent No. 5,079,172 (Hari et al.) discloses a method and kit for detecting the presence of antibodies using gold-labeled antibodies. Microspheres coated with an antigen reactive with the first antibody are reacted with the first antibody from serum or other sources. The gold-labeled antibody is reacted with the first antibody antigen complex on the microsphere and detected. Preferably, the gold particles are detected using an electron microscope.

PCT/US95/04547 describes the use of soluble submicron particles (dendrimers) that are labeled with antibodies to coat membranes. The assay procedures described are flow through EIA and fluorescence immunoassays requiring washing steps in order to obtain a response.

Currently, most lateral flow and flow-through gold immunoassays utilize antibody bound directly to porous membranes or to particles such as glass or latex to capture antigen-antibody- gold colored complexes.

The original tests developed by New Horizons Diagnostics utilized primarily 20 nm gold particles to tag the antibodies. This resulted in a sensitivity level of about 50 ng for botulism toxin and around 10_5 organisms/cc for the quantity of specific bacteria that could be detected. 20 nm particles were used initially because of the stability of the gold tagged antibody conjugate and the low background signal (if any) it gave in negative test samples. Ideally, it is better to label with a much larger gold colloid (anywhere between 50 run to 100 nm) to increase the sensitivity of test results. However, the problem with using larger colloidal gold complexes for tagging has been the increase in non-specificity, which creates false positive reactions.

SUMMARY OF THE INVENTION

The present invention proposes a much more sensitive immunoassay test, which is easier to use and interpret. The entire test is conducted on a test strip, and the detection antibody is preferably a FAB fragment that has been labeled with a 50 - 100 nm gold particle and immobilized on a test pad. The invention provides a simplified, sensitive and specific test procedure for the determination and detection of an immunologically reactive analyte in an aqueous sample.

Specifically, the present invention discloses a capture membrane to align and secure capture antibodies on a solid surface so that the immunological activity of the capture antibody is not sterically hindered. Consequently, optimal binding capacity is achieved, resulting in a minimum loss in binding activity between the capture antibody and the ligand. In one preferred embodiment, dendrimers are used to secure the capture antibodies to a test strip upon which a sample is placed.

In another embodiment of the invention, the site of the capture membrane is protein A or protein G. Alternatively, the capture membrane site may be lectin receptors, to which no capture antibodies are applied. Antispecies antibodies may be used, particularly in combination with the dendrimers or protein G.

The present invention also discloses an improved gold immunoassay test system which uses larger gold colloids for tagging antibodies (anywhere between SO

nm to 100 nm) to increase the sensitivity of test results, (i.e. each specific antibody that reacts, delivers more gold complex to the antibody capture membrane) without any residual non-specific activity. The elimination of non-specific background activity problems results from the use of blocking chemistries which inhibit non-specific reactions without altering the desired specific reaction and the use of specifically designed recombinant FAB antibodies.

FAB antibodies are unlike whole antibodies in that their FC or constant regions are eliminated. It is this region of the antibody molecule that often causes problems with non-specificity.

In this invention, the FAB antibodies for detecting a positive result are attached to gold colloidal masses, in the range of 50 - 100 nm. These gold-FAB antibody complexes are positioned on a test strip, downstream from where the antigenic sample is applied.

The gold FAB-antibodies may be attached to microspheres, thereby allowing for more antibodies to be located at the reagent site.

Additionally, other metals or dyes may be attached to the FAB antibodies, in place of the gold particles.

Further downstream from both the antigenic or ligand sample and the gold-FAB sample is a set of antibodies, specific for the gold-FAB antibody-antigen or antibody-ligand complex. These antibodies serve to concentrate the complex in one location, thereby allowing for a red stripe to appear on a set section of the test strip when there is a positive reaction.

The capture antibodies are located further downstream from the reagent and the sample site.

Dendrimers are one way of attaching the capture antibodies to the test strip. Dendrimers are three dimensional, tree-like polymers. The dendrimers have a small size, good solubility, higher segmental densities, interior void space, and lower viscosities. Dendritic polymers can be constructed by both divergent and convergent synthetic methods. The divergent synthesis starts from a center core, and then grows each layer in a stepwise fashion, while the convergent method

assembles exterior end groups and dendrons first before being coupled onto a core. Each re-iteration or layer is defined as a generation. The more layers there are in the dendrimeric structure, the more rigid the dendrimers molecule itself becomes. Up to about G8, a dense packing stage also occurs, where beyond this point, monomers can no longer react with every surface group quanitatively due to the stearic effect. In the case of immunoassays, the rigid, spherical protein-like sizes controls the antibody binding direction, while the exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion on a membrane surface. This property allows the dendrimers-antibody conjugate to self-assemble to its best binding orientation. As a result, the sensitivity and reproducibility of the immunoassay in the detection of different bio-threat agents has been significantly improved, while the assay time has been shortened. In addition due to the low viscosity nature and self assembling capabilities of dendrimers-antibody conjugates, the process for producing these tickets has been simplified, and has reduced lot to lot variability.

Dendrimers have the advantage, that they can be synthesized with an exactly uniform molecular weight, whereas the conventional polymers always have a particular molecular weight distribution. In dendrimers with particular functinal groups can be manufactured with a defined number of such reactive groups.

The coupling of antibodies or other ligands to outer surfaces of dendrimers (for example polyamidoamine [PAMAMS] dendrimers) can be done by various well known chemistries which describe Carbonsulfur, carbon-oxygen, and carbon-nitrogen coupling procedures. The exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion onto the surface of a membrane. The structural composition of the dendrimer controls the spatial arrangement of the attached antibody molecules. This assures the optimal binding activity of the immobilized capture antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and advantages of the present invention will become more readily apparent from the following description, reference being made to the accompanying drawings in which:

FIG. 1 is an overall view of the immunoassay test system;

FIG. 2 is an exploded view of the immunoassay test system;

FIG. 3 is a side view of the immunoassay test strip;

FIG. 4 is an overhead view of the immunoassay test strip; and

Fig. 5 is a schematic drawing of the antigenic-antibody reaction.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Referring to Figures 1-5, the immunoassay test system 1 comprises an enclosure 2, which is preferably plastic. This plastic enclosure comprises a top section 3 and a bottom section 4 which are held together by male 5 and female 6 peg joints.

The top section 3 of the enclosure 2 has an opening 7 for placing a sample. There is also an opening 8 to visualize the test results 9 and the control results 10.

The bottom section 4 comprises a tray 11 into which fits a test strip 12. The test strip 12 preferably has a membrane support 13. The membrane support 13 may be comprised of plastic, cardboard, or any other rigid material. On top of the membrane support 13 is a testing layer 14, preferably made out of nitrocellulose. On top of the nitrocellulose or testing layer 14 are the areas to which the appropriate reagents or samples are applied or affixed. The nitrocellulose/testing layer is affixed to the membrane support 13 by an adhesive 31.

At one end of the test strip 12 is the sample site 15 to which the sample is to be applied. This sample site 15 may have another nitrocellulose or adsorbent sample pad 23 residing on top of the testing layer, to which the sample is transferred. The sample may be in the form of an antigen or ligand 16 in a fluid.

The list of target ligands and anti-ligands which potentially may be detected or determined includes antigens and ligands found in animal body fluids, as well as

antigens associated with bacteria, parasites, fungi, viruses, toxins, anthrax, etc. Moreover, therapeutic drugs and controlled substances having small molecules, such as, for example, theophylline, may be detected or determined using the present invention.

The sample travels downstream from the sample site 15 to the gold immunoassay site 18 where FAB antibody coated gold sol particles 19 reside. The gold particles 19 attached to the FAB antibodies 20 are preferably larger than 20 nm, more preferably in the range of about 50 to 100 nm, and most preferably in the range of from about 70 to 90 nm. Larger particles may also be used wherein a number of FAB antibodies 20 are attached to the gold particle 19. The gold sol labeled FAB antibodies 21 are dried and deposited on the strip 12.

The metal sol particles to be used in accordance with the present invention may be prepared by coupling an immunologically reactive substance directly to the gold particle. Additionally, the labeled component may be prepared by coupling the substance to the particle using a biotin/avidin linkage. In this latter regard, the substance may be biotinylated and the metal containing particle coated with an avidin compound. The biotin on the substance may then be reacted with the avidin compound on the particle to couple the substance arid the particle together. In another alternative form of the invention, the labeled component may be prepared by coupling the substance antibody to a carrier such as bovine serum albumin (BSA), and using this to bind to the metal particles.

The metal sol particles to be used in accordance with the present invention may be prepared by methodology which is well known. For instance, the preparation of gold sol particles is disclosed in an article by G. Frens, *Nature,* 241, 20-22 (1973). Additionally, the metal sol particles may be metal or metal compounds or polymer nuclei coated with metals or metal compounds, as described in U.S. Pat No. 4,313,734. Other methods well known in the art may be used to attach the gold particles to the FAP antibodies. The metal sol particles may be made of platinum, gold, silver, or copper or any number of metal compounds which exhibit characteristic colors.

Similarly, the antibodies do not necessarily have to be attached to a metal sol particle, but may instead be attached to a dye with an extinction coefficient equal to or greater than gold. The metal sol particles or dyes should have a high extinction coefficient equal to or greater than gold.

There are a number of ways in which the gold labeled FAB antibodies 21 may be deposited on the strip 12.

In an alternative and preferred embodiment, the gold labeled FAB antibodies are deposited and dried on a rectangular or square or adsorbent FAB antibody pad 22, the pad preferably about .25" x .25" or less. This FAB antibody pad 22 is positioned downstream from where the sample is applied on the strip 12. Preferably, the FAB antibody pad 22 fits underneath the distal end 24 of the sample pad 23.

In yet another embodiment of the invention, the antibodies are attached to microspheres. This has the effect of increasing the number antibodies in a given area.

The process for attaching the antibodies to the microsphere(s) begins with the use of protein reactive microspheres (MX-Covaspheres^{R} of diameter .5 micrometers or .9 micrometers purchased from Duke Scientific Corporation, Pal Alto, California 94303. The microspheres may also be purchased from other suppliers, as well). The binding is at the amino groups of the protein. They were then coated with various antibodies. A suspension of the spheres are mixed after sonication with the antibodies in water or in a phosphate buffer solution, after which they are incubated at room temperature for 10-75 minutes. The mixture is then centrifuged in an Eppendorf microcentrifuge and the pellets containing the antibody-linked microspheres are suspended in a buffer containing 1-5% by wt/volume bovine serum albumin (BSA) for 1 hour at room temperature. The BSA blocked any unreacted surfaces of the microspheres. After one more centrifugation, at 10,000 for 10 minutes, the spheres were resuspended in the above buffer (TBS with 5% BSA) and stored at 4 degrees C. before using.

The solid phase particles may comprise any one of known, water dispersable particles, such as, for example, the polystyrene latex particles discloses in U.S. Patent No. 3,088,875. Such solid phase materials simply consist of suspensions of small, water-insoluble particles to which antibodies are able to bind. Suitable solid phase particles are also disclosed, for example, in U.S. Patent Nos. 4,184,a49; 4,486,530; and 4,636,479.

The solid phase particles useful in connection with the invention may comprise, for example, particles of latex or of other support materials such as silica, agarose, glass, polyacrylamides, polymethyl methacrylates, carboxylate modified latex and Sepharose. Preferably, the particles will vary in size from about 0.2 microns to about 10 microns. In particular, useful commercially available materials include .99 micron carboxylate modified latex, cyanogen bromide activated Sepharose beads (Sigma) , fused silica particles (Ciba Corning, lot #6), isothiocyanate glass (Sigma), Reactogel 25DF (Pierce) and Polybead - carboxylate monodisperse microspheres. In accordance with the invention, such particles may be coated with a layer of FAB antibodies coupled thereto in a manner known per se in the art to present the solid phase component.

If the sample contains an antigen or ligand 16 to which the gold FAB antibodies 21 react, there is a antigenic-antibody bonding between the sample and the gold FAB antibodies 21. The antigen-gold FAB antibody complex 25 continues to migrate along the capture site 26 where another antibody is attached to a region of the strip 12.

The antibodies 27 supported by the dendrimers 32 are designed to react specifically to the antigen, effectively forming an antibody-antigen-gold FAB antibody sandwich 29 if there is a positive reaction. If there is a negative reaction, no "sandwich" is formed, and the unreacted ligand proceeds to the end of the strip 12 wherein an absorbent pad 30 absorbs the fluid and unreacted labelled-antibody proceeds to the end of the strip 12.

To prepare the capture site 26, antibodies are bound to dendrimers prior to their placement on the strip 12. This is then layered on the strip and dried.

In performing the testing of an antigen or ligand, it should be noted that if there is a positive test result, the antigens or ligands will attach to the gold FAB antibodies as it migrates from the sample site, whereupon the antigens or ligands will attach to the antibodies attached to the dendrimers. At this point, the concentrated appearance of the gold particles appears as a red to purple line. If however, the ligands do not attach to the gold labeled antibodies, the antibodies will not be bound to the capture site 26.

There are a number of other means in which antibodies may be attached or positioned at the capture site. instead of using dendrimers at the capture site, Protein A and/or Protein G from Staphylococcus cell wall may be deposited by conventional means to the test strip. Because of the unique chemistry of Protein A and/or Protein G, the capture antibodies laid down at the capture site are aligned such that the active or binding ends are facing outward from the protein surface. Protein A and Protein G have a strong anti-FC activity. Consequently, FAB antibodies tagged with gold must be used at the reagent site; otherwise, false positive reactions may result as "loose" gold antibodies migrate along the teat strip.

In another embodiment of the invention, a species-antispecies antibody combination is laid down on the test strip. For example, a goat antirabbit antibody is laid down on the test strip. The rabbit antibody is attached or bound to the goat anti-rabbit antibody. The rabbit antibody may be non-specific, so that any antigen that migrates along the teat strip will be captured by the rabbit antibody. If the colorimetric tagged antibody is attached to the antigen, a positive result will be appear in the form of an indicator line. It should be noted that the anti-species antibody may be attached to the test strip by a dendrimeric arrangement.

In yet another alternative embodiment of the invention at the capture site is to have lectin receptors. This lectin layer will bind the antigen as it migrates along the length of the test strip. In the preferred embodiment, the lectin may be bound to a dendrimer to optimize its binding capability.

The superior sensitivity of this test format allows for detection of amounts of antigen or ligand, measured in micrograms.

Additionally, this test can also have positive and negative control lines. The positive control line has an anti-Fab substance or antibody laid down at the appropriate spot on the strip, downstream from the sample test site and from the FAB reagent site. This line should always appear when FAB antibodies are used in the test. If the positive control is negative, then the test is invalid.

The negative control can use any nonrelated antibody to coat the strip. There should be no capture of the antigen or ligand by the non-related antibody. If the negative control is positive, i.e., a line appears, than the test is invalid.

Many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood within the scope of the appended claims the invention may be protected otherwise than as specifically described.

WE CLAIM:

1. A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a testing layer, said testing layer comprised of an absorbent material;

a sample site at one end of the testing layer, to which a ligand is applied;

a reagent-colorimetric immunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of antibodies attached to colorimetric particles thereby forming colorimetric labeled antibodies, said colorimetric labeled antibodies residing at said reagent-colorimetric immunoassay site;

a capture site, said capture site comprising means to retain ligand-colorimetric labeled antibody complexes in optimal alignment, said means comprising capture antibodies and binding means to align said capture antibodies so that active ends of said capture antibodies face away from an outwardly facing surface of said testing layer;

wherein said ligand, when placed on said sample site, migrates to the reagent-colorimetric-immunoassay site, and then migrates to the capture site, such that if said ligand reacts positively with the colorimetric labeled antibodies, said ligand-colorimetric labeled antibody complexes migrate to the capture site, and a positive response will appear as a colorimetric line at the capture site when an antibody-ligand-colorimetric labeled antibody sandwich is formed.

2. The immunoassay test system of claim 1, wherein said antibodies at said reagent-colorimetric immunoassay site are FAB antibodies.

3. The immunoassay test system of claim 1, wherein said testing layer is comprised of nitrocellulose.

4. The immunoassay test system of claim 1, further comprising a support backing layer upon which the testing layer resides.

5. The immunoassay test system of 4, wherein said support backing layer is made of a rigid material, said material being selected from the group consisting of cardboard or plastic.

6. The immunoassay test system of claim 1, wherein said testing layer is fixed to said backing layer by an adhesive.

7. The immunoassay test system of claim 1, wherein said colorimetric particles are selected from the group consisting of gold particles, silver particles, platinum particles, copper particles and encapsulated dyes.

8. The immunoassay test system of claim 7, wherein the colorimetric particles are gold sol particles.

9. The immunoassay test system of claim 8, wherein said gold sol particles may range in size from about 20 ng to about 100 ng.

10. The immunoassay test system of claim 9, wherein said gold sol particles may range in size from about 50 ng to about 90 ng.

11. The immunoassay test of claim 1, further comprising microspheres, wherein said colorimetric labeled antibodies are attached to said microspheres.

12. The immunoassay test system of claim 1, wherein said binding means which aligns said capture antibodies is selected from the group consisting of Protein A, Protein G, and any other substance which binds said antibodies at said capture site.

13. The immunoassay test system of claim 1, wherein said antibodies at the reagent-colorimetric immunoassay site are FAB antibodies.

14. The immunoassay test system of claim 13, wherein said binding means which aligns said capture antibodies is selected from the group consisting of Protein A and Protein G.

15. The immunoassay test system of claim 1, wherein said ligand is an antigen.

16. The immunoassay test system of claim 1, wherein the ligands and anti-ligands which may be detected are selected from the group consisting of antigens and ligands found in animal body fluids, antigens associated with bacteria parasites, fungi, viruses therapeutic drugs and controlled substances having small molecules.

17. The immunoassay test system of claim 12, wherein said dendrimers for aligning said capture antibodies are starburst dendrimers.

18. The immunoassay test system of claim 1, further comprising an enclosure,

said enclosure comprising a top section, said top section having openings for placing a sample and for visualizing the test results; and

a bottom section said bottom section having a tray into which fits a test strip.

19. The immunoassay test system of claim 18, wherein said enclosure is comprised of plastic.

20. The immunoassay test system of claim 1, wherein said absorbent material allows for absorption of a liquid sample and has capillary action capability, thereby allowing the sample to migrate along the length of the test strip.

21. The immunoassay test system of claim 1, further comprising a sample pad positioned on top of the testing layer at the sample site upon which the sample is added.

22. The immunoassay test system of claim 1, further comprising a FAB antibody pad positioned on top of the testing layer at the reagent-gold immunoassay site at which the FAB antibodies may react with an appropriate ligand.

23. The immunoassay test system of claim 1, further comprising an absorption pad positioned on top of the testing layer downstream from the capture site to absorb any excess fluid.

24. The immunoassay test system of claim 1, further comprising a negative control line positioned on said test strip, such that if said negative control line shows a positive result in the performance of a test, said test is invalid.

25. The immunoassay test system of claim 1, further comprising a positive control line positioned on said test strip such that if said positive control line shows a negative result in the performance of a test, said test is invalid.

26. The immunoassay test system of claim 1, further comprising a positive control line and a negative control line positioned on said test strip such that if said positive control line shows a negative result in the performance of a test, or said negative control line shows a positive result in the performance of said test, said test is invalid.

27. A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a support backing layer;

a testing layer, said testing layer comprised of an absorbent material;

a sample site at one end of the testing layer, to which a ligand is applied;

a reagent-colorimetric immunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of FAB antibodies attached to colorimetric particles thereby forming colorimetric labeled FAB antibodies, said colorimetric labeled FAB antibodies residing at said reagent-colorimetric immunoassay site;

a capture site, said capture site comprising means to retain ligand-colorimetric labeled antibody complexes, said means to retain said ligand-colorimetric labeled antibody complexes being a lectin layer attached to said test strip;

wherein said ligand, when placed on said sample site, migrates to the reagent-colorimetric-immunoassay site, and then migrates to the capture site, such that if said ligand reacts positively with the colorimetric labeled FAR antibodies, said ligand-colorimetric labeled FAR antibody complexes migrate to the capture site, and a positive response will appear as a red line at the capture site when an antibody-ligand-colorimetric labeled FAR antibody sandwich is formed.

28. A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a support backing layer;

a testing layer, said testing layer comprised of an absorbent material;

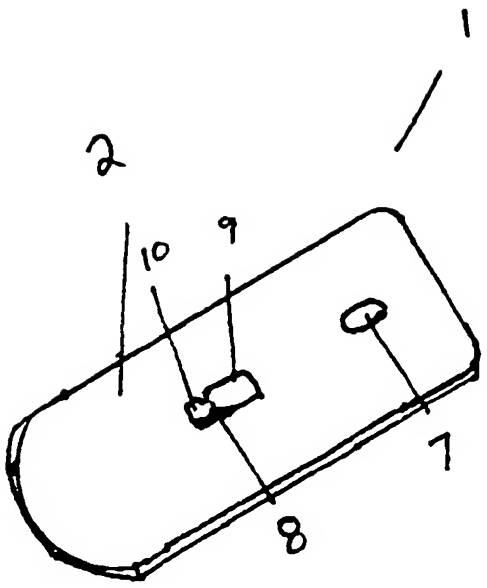
a sample site at one end of the testing layer, to which a ligand is applied;

a reagent-colorimetric immunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of antibodies attached to colorimetric particles thereby forming colorimetric labeled antibodies, said colorimetric labeled antibodies residing at said reagent-colorimetric immunoassay site;

a capture site, said capture site comprising means to retain ligand-colorimetric labeled antibody complexes in optimal alignment, said means to retain said ligand-colorimetric labeled antibody complexes being a species antibody-antispecies antibody layer attached to said test strip, said capture site further comprising a means of attaching the antispecies antibodies to said test strip, wherein said species antibodies are reacted and attached to said antispecies antibodies; and

wherein said ligand, when placed on said sample site, migrates to the reagent-colorimetric-immunoassay site, and then migrates to the capture site, such that if said ligand reacts positively with the colorimetric labeled antibodies, said

ligand-colorimetric labeled FAR antibody complexes migrate to the capture site, and a positive response will appear as a red line at the capture site when an antispecies antibody species antibody-ligand-colorimetric labeled antibody sandwich is formed.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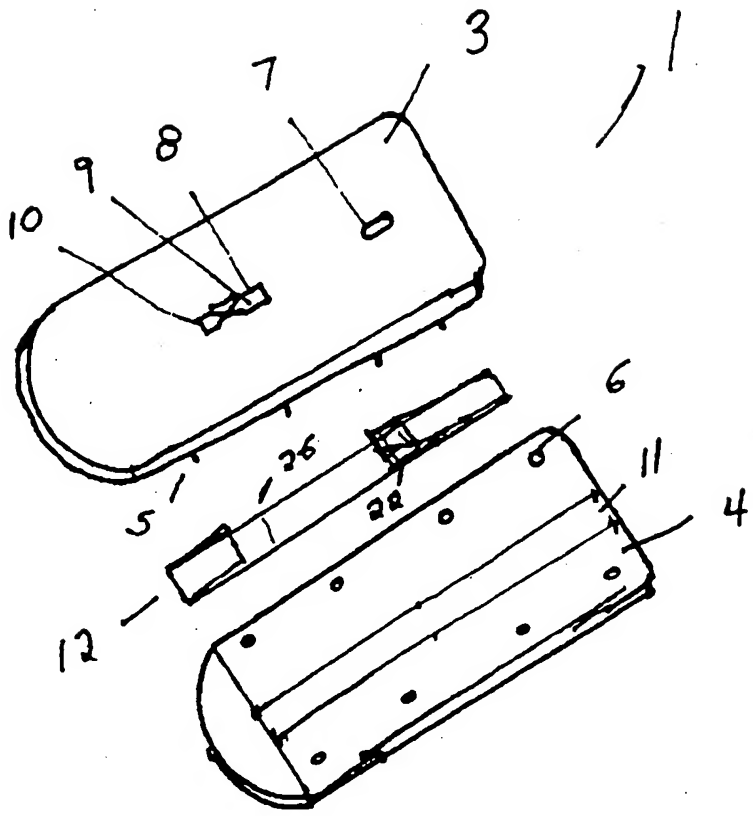
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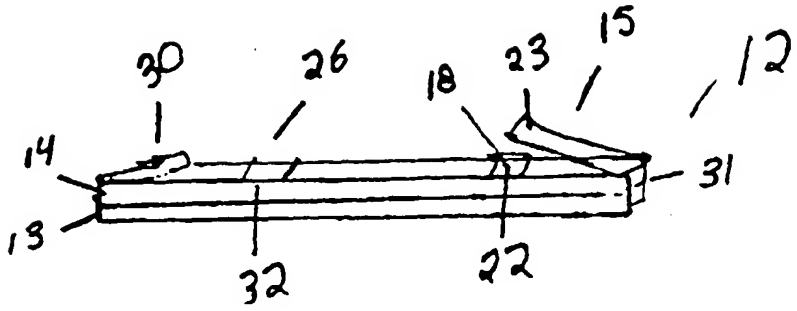
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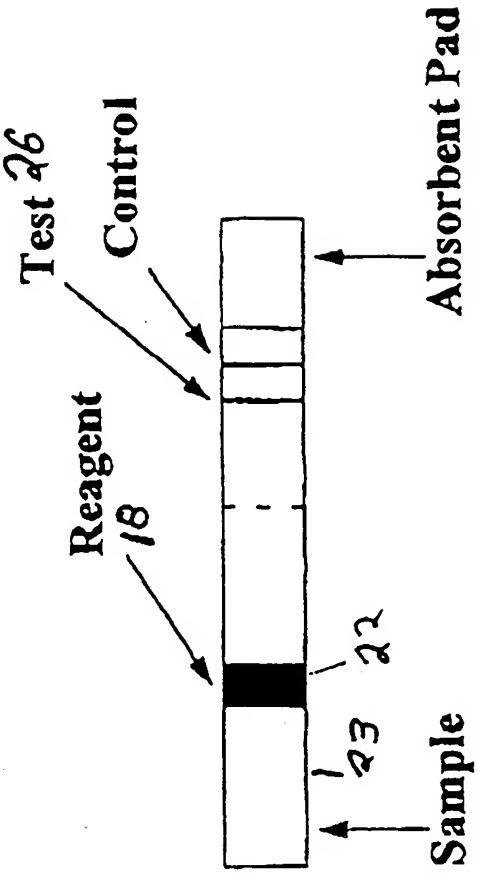
Lateral Flow Device



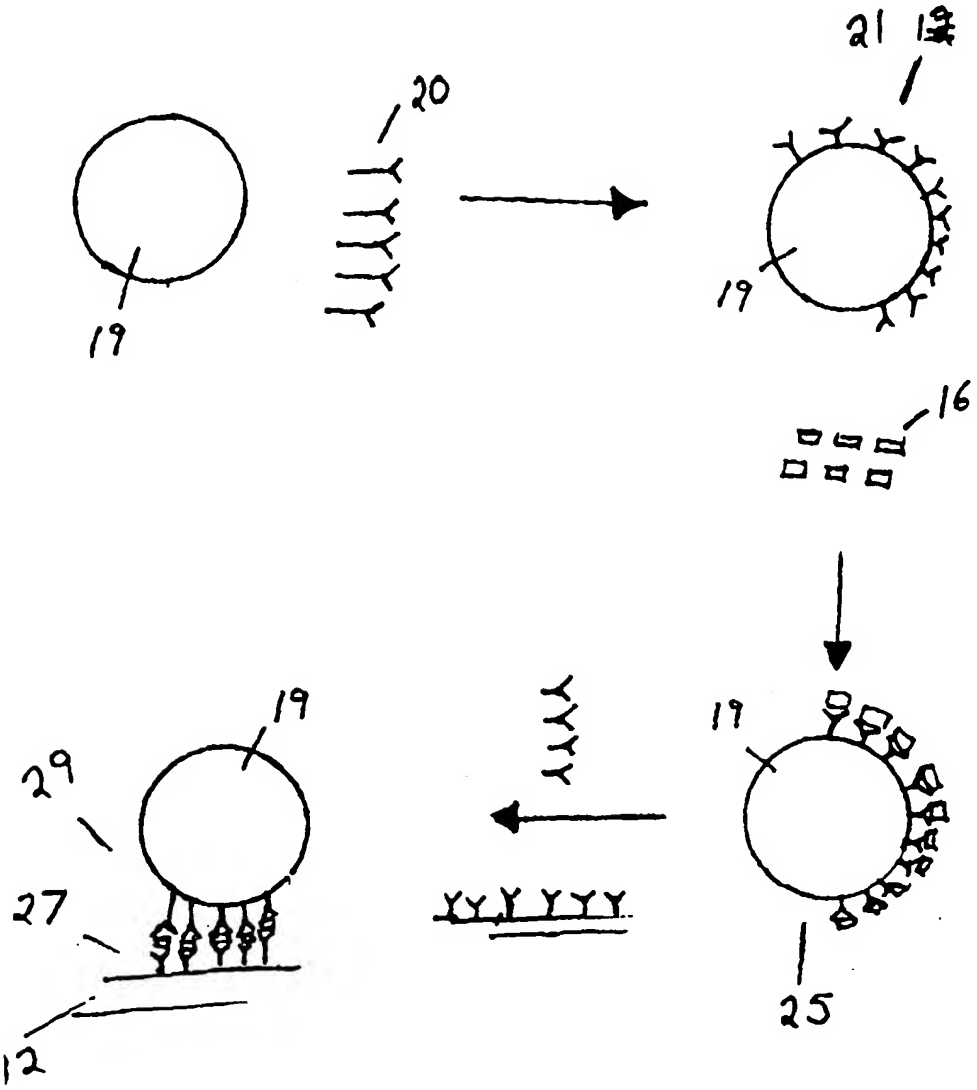


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13284

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/558
US CL :436/514

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,602,040 A (MAY et al) 11 February 1997, see entire document.	1-28

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
12 SEPTEMBER 1999	19 OCT 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13284

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

422/55-58, 61;
435/5, 7.1, 7.2, 7.21, 7.22, 7.32, 287.1, 287.2, 287.7, 287.8, 805, 810, 970;
436/164, 169, 512, 514, 518, 525, 531, 533, 534, 805, 810